Discovery of an Abnormal Lignin in a Loblolly Pine Mutant

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Introduction

Lignins are complex phenolic plant polymers essential for mechanical support, defense, and water transport in vascular terrestrial plants. They are usually derived from three hydroxycinnamyl alcohol precursors **2a-c** in varying proportions, Figure 1. Low levels (~5%) of cinnamaldehydes and benzaldehydes are found in all isolated lignins and are responsible for the bright crimson staining of lignified tissues by phloroglucinol/HCl. Removal of lignin from wood and plant fibers is the basis of chemical pulping to produce diverse pulp and paper products. Genetic engineering of the lignin biosynthetic pathway to lower lignin concentration or construct lignins more amenable to extraction is an active area of current research.

CAD catalyzes the last step of the lignin precursor biosynthetic pathway (Fig. 1), reduction of hydroxycinnamaldehydes **1** to hydroxycinnamyl alcohols **2** (the conventional lignin monomers or monolignols). A reduction in CAD activity might lead to accumulation of hydroxycinnamaldehydes **1** which could copolymerize with normal lignin monomers. Transgenic plants, suppressed in the synthesis of CAD sometimes have red-brown xylem tissue, resembling that of grass brown midrib mutants. Such plants have increased aldehyde levels, although little of the aldehyde may actually be incorporated into the lignin.

Here we report that a viable loblolly pine, homozygous for the mutant *cad-n1* allele, incorporates novel monomers into its lignin in response to a CAD deficiency. The lignin structural changes were extensive and not predicted by the current view of the lignin biosynthetic pathway. The wood of this mutant is brown-red, similar to the color of the xylem in *brown midrib* mutants and transgenic plants suppressed in lignin biosynthetic enzyme activity. CAD activity is 1% or less of wild type, and relative abundance of *cad* mRNA transcript is greatly decreased. In mutant plants, free coniferaldehyde **1b** (the CAD substrate) accumulates to a high level.

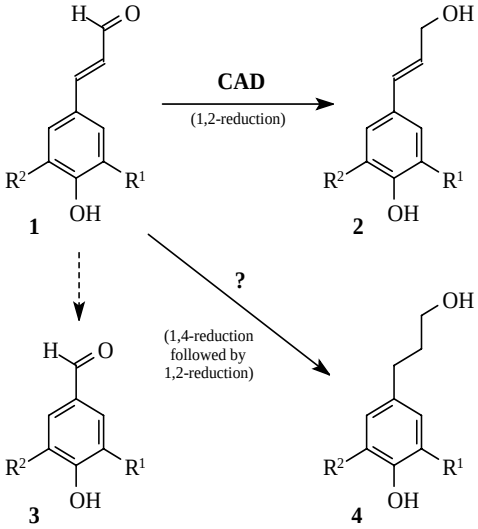

*Figure 1. Some precursors and products involved in the lignin biosynthetic pathway. The normal lignin monomers are the p-hydroxycinnamyl alcohols **2**; p-coumaryl alcohol **2a**, coniferyl alcohol **2b** and sinapyl alcohol **2c**. Coniferaldehyde **1b** is normally reduced regioselectively to produce coniferyl alcohol **2b**. When CAD activity is depressed, coniferaldehyde **2b** accumulates and could polymerize or co-polymerize into lignin. Dihydroconiferyl alcohol **4b**, observed previously only as a minor component of softwood lignins, is presumed to derive from coniferaldehyde **1b** via a 1,4- followed by a 1,2-reduction. However, no mechanism for this conversion has been reported. p-Coumaryl alcohol **2a** is readily derived from its aldehyde **1a** in the mutant, implying that different CAD enzymes are involved for **1a**→**2a** vs. **1b**→**2b**.*

Results and Discussion

Milled wood lignins were isolated for NMR analysis from the wood of a 12 year old CAD-deficient mutant and a normal sibling from the same cross. An estimate of the subunit composition of this unusual lignin fraction, based on quantitative NMR and other analytical data, is given in Table 1.

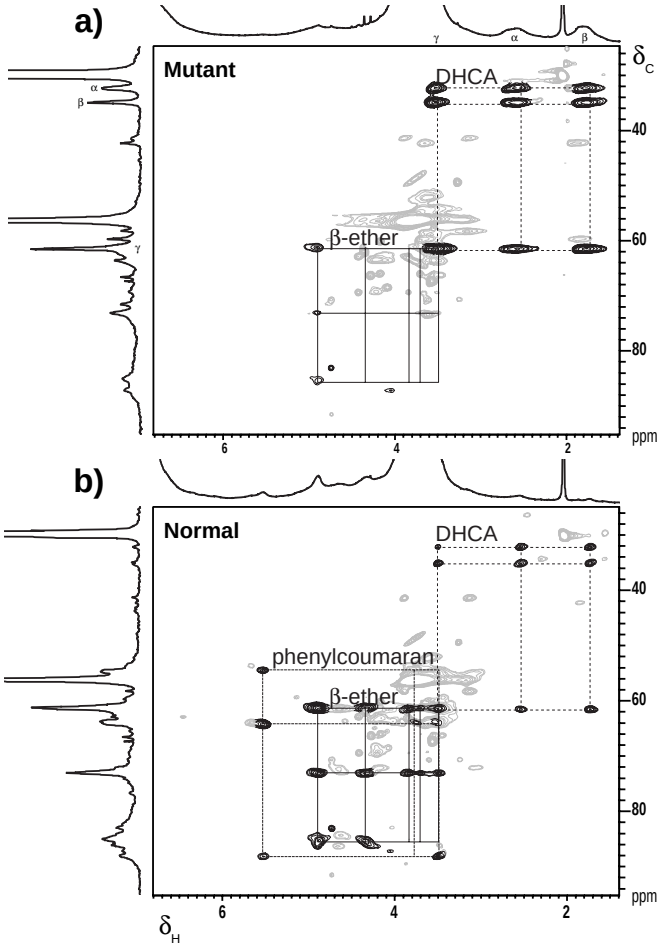

Figure 2. Regions of the HMQC-TOCSY spectra of milled wood lignins from a) the pine cad-n1 mutant, b) a cad-normal wood. In the CAD mutant, dihydroconiferyl units are dominant, displacing much of the intensity from the normal coniferyl alcohol-derived region. Some of the minor units can be seen in the pine samples when looking at lower contour levels (not shown). The normally predominant b-aryl ether and phenylcoumaran components, Figure 2b, are severely reduced in the cad-n1 mutant, with only some b-ether peaks being observable at comparable contour levels — these may also arise from p-coumaryl alcohol (in addition to coniferyl alcohol). Grey contours are from intense methoxyl signals, carbohydrate impurities, and other lignin structures not discussed here.

NMR spectra show that both coniferaldehyde and vanillin endgroups, as well as new aldehydes, are present in the lignin of the pine mutant as may be expected from the suppression of CAD. Wood from the mutant also had a higher extractable aldehydes content. The most striking feature of spectra was due to unexpected elevation of dihydroconiferyl alcohol units. The HMQC-TOCSY experiment (Fig. 2) identified the coupling network for the aryl propanol sidechain that are consistent with model compound data. Products representing hetero-coupling of dihydroconiferyl alcohol with a conventional lignin monomer/oligomer as well as products from initial 5–5-homo-coupling of dihydroconiferyl alcohol monomers are present in roughly equal amounts reinforcing the claim that dihydroconiferyl alcohol is a major monomer during lignification. The monomer was also found in wood extracts.

CAD normally effects a regioselective "1,2-reduction" (at C-9) of coniferaldehyde **1b** to produce coniferyl alcohol **2b**. Our results suggest that the loss of CAD activity activates or up-regulates pathways based on "1-4 reduction" (at C-7) and subsequent 1,2-reduction during lignin formation to produce the dihydroconiferyl alcohol monomer **4b** (Fig. 1). If the biochemical reduction is not totally regioselective, the small amounts of **4b** producing the dihydroconiferyl units seen in normal pine lignins could be explained but this rationale would not allow production of **4b** in such major proportions without a significant shift in enzyme activity or without enhanced activity of an alternate enzyme. At least one new enzyme would be required to explain these results.

The amount of subunits derived from *p*-coumaryl alcohol **2a** in the mutant is unchanged (Table 1), while the amount of coniferyl alcohol subunits **2b** is greatly reduced. These results imply that the formation of *p*-coumaryl alcohol **2a** utilizes an independent mechanism such as an additional enzyme with "1,2-reductase activity" specific for *p*-hydroxycinnamaldehyde **1a**. Furthermore, few dihydro-*p*-coumaryl alcohol **4a** units were detected (29). The 1,4-reductase activity proposed for the formation of dihydroconiferyl alcohol is therefore equally specific for coniferaldehyde **1b**.

Incorporation of novel monomers into lignin is inconsistent with a high level of enzymatic specificity in lignification. Independence from rigid enzymatic control is further supported by other examples of incorporation of non-traditional monomers into lignins — see page 30.

Conclusions

Well characterized differences in lignin subunit composition have long been known between major

taxonomic groups of higher plants, for example, between lignins of hardwood and softwood trees. However, the narrow range of variation in lignin compositions within groups has suggested structural constraints imposed for vascular function and support. The ability of this pine mutant to produce a functional lignin polymer from unexpected subunits extends the limit of "metabolic plasticity" for the formation of lignin within an individual species. Concepts of lignin function based on the previous range of lignin compositions must now be reexamined in view of the unusual structure and composition of lignin in this mutant pine. A greater understanding of these processes should increase our opportunities to modify lignin content or composition through genetic engineering — see "A New Frontier for Plant Modification" page 27 in these summaries.

For a more complete manuscript describing these findings in greater detail, see *Science*, **277**:235-239, 1997. The full manuscript in pre-publication form is on our website at: *http://www.dfrc.wisc.edu/FullTextPubs.html*

Table 1. Estimates of subunit compositions (from quantitative ^{13}C-NMR and DFRC-method data) of the normal and mutant pine isolated lignins. **2a** = *p*-coumaryl alcohol units; **2b** = coniferyl alcohol units; **1** = cinnamaldehyde units; **3** = benzaldehyde units; * aldehydes at ~188 ppm in ^{13}C-NMR spectra appear to derive from coniferaldehyde; **4b** = dihydroconiferyl alcohol (+ traces of dihydro-*p*-coumaryl alcohol **4a**), the major component of the *cad-n1* mutant lignin.

Lignin	**1**	**2a**	**2b**	**3**	**4b**	*****
cad-n1-mutant	15	10	15	15	30	15
cad-normal	7	10	73	7	3	trace